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TITLE: The Role of the Polypyrimidine Tract Binding Protein on CD44 Alternative Splicing in Breast Cancer

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The cytogenetic and nuclear changes that occur during tumor progression in the breast have been well documented, but the causes of these alterations are poorly understood. Changes in estrogen receptor status, loss of responsiveness to conventional chemotherapy, gain in metastatic potential, accumulation of PNCs, and differential splicing of numerous genes are examples of changes seen in breast cancer cells during tumor progression. Thus far, a strong connection between the splicing machinery and these subtle, yet significant, changes in gene expression has yet to be documented. Likely candidates are the alternative splicing factors most notably the Polypyrimidine Tract Binding Protein (PTB). PTB is a known repressor of exon definition. During breast cancer progression, we believe, the ability of PTB's ability to repress exons is altered. In order to understand the changes in PTB function as cancer cells de-differentiate, a clear understanding of PTB mechanism must be attained. We are using the regulation of FGF-R2 exon IIIb as a model system to study PTB mediated exon repression. Thus far, we have mapped numerous binding sites for PTB and found them to be important in the repression of this exon. Furthermore overexpression of PTB and heterologous recruitment of PTB result in the repression of this exon. RNAi mediated PTB depletion ~~?~~results in the increase in exon IIIb inclusion.

14. SUBJECT TERMS
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Introduction

The broad long-term goal of this research is to understand the mechanism of alternative exon repression mediated by the polypyrimidine tract binding protein (PTB). Little is known about this form of gene expression regulation in general and even less is known about how this regulation is lost in the progression of cancers. The initial specific aims of this grant were geared toward understanding the mechanism of PTB action on the alternative splicing of CD44. These aims have since been amended (see Appendix I.) to focus still on PTB mediated exon repression, however, now the gene of interest is the fibroblast growth factor receptor 2 (FGF-R2). The spirit of this research, however, is the same for we believe that the molecular process that PTB is involved in to repress alternatively spliced exons is shared by many genes (Wagner & Garcia-Blanco, 2001). It has also recently been shown that deletion of the FGF-R2 exon IIIb, the exon that we have found to be repressed by PTB, resulted in deficiencies in normal mammary gland development (Mailleux et al, 2002). Furthermore, a subset of breast cancer cell lines has been found to possess an increase in FGF-R2 (IIIb) mRNA (Heiskanen et al, 2001). Hence, although the focus of this research is on PTB regulation in alternative splicing, the new gene of interest appears to be directly pertinent to the field of breast cancer.

Body

In the annual report summarizing the first year of this funded period, fluorescent reporters capable of recapitulating *in vivo* exon silencing of FGF-R2 exon IIIb were described. These data were the goal of task 1c. of the statement of work. In the past twelve months of research much progress has been made to demonstrate the polypyrimidine tract binding protein (PTB) as an antagonist of exon definition. These achievements, which are described both below and in the appendix, include demonstrating that PTB is bound both upstream and downstream of FGF-R2 exon IIIb and that these binding sites are indeed important for silencing exon IIIb inclusion. In addition, we have demonstrated that artificial recruitment of PTB to heterologous binding sites flanking exon IIIb can also elicit silencing , thus recruitment of PTB is sufficient to cause exon IIIb inclusion. Most importantly, we have used RNA interference (RNAi) to deplete PTB *in vivo* and shown that this depletion leads to both an increase of exon IIIb inclusion in minigene constructs as well as the endogenous FGF-R2 mRNA. These data prove the initial hypothesis of this research proposal that PTB is a repressor of exon inclusion. Furthermore, these data were the goals of both task 1 and most of task 2 in the revised Statement of Work (see appendix).

In order to implicate PTB as a antagonist of FGF-R2 exon IIIb definition two initial discoveries had to be made: 1) that there are indeed intronic silencers repressing IIIb inclusion and 2) that PTB binds to these regions. Demonstration of the first point is in figure 1. Minigenes were created to delete the previously identified upstream intronic splicing silencer (UISS), which was found to bind PTB, as well as deletions with the intronic control element (ICE) (Fig 1A) and were transfected into DT3 and AT3 cells. These minigenes were found to have an increase in FGF-R2 exon IIIb inclusion (Fig 1b). Substitution of the ICE with an

irrelevant sequence also resulted in an increase in exon IIIb inclusion (Fig 1c). Placement of the ICE downstream of unrelated exons (in this case both exon 8 of FGF-R3 as well as exon 5 of cardiac troponin T in Fig 1d) demonstrated that the ICE was sufficient to repress some exons but not all (Fig 1E). Sequence analysis of the ICE showed that within this 220 nt region was seven evolutionarily conserved UCUU motifs, which have been found to be putative PTB binding site (see Wagner & Garcia-Blanco, 2001) as well as another conserved stretch of nucleotides bearing no resemblance to other known cis elements. Deletion analysis of the ICE downstream of FGF-R2 exon IIIb demonstrated that these UCUU motifs were in fact involved in the repression of exon IIIb (Fig 2 and 3). These motifs were indeed found to bind to PTB (Appendix IV. Fig 1). We have also found that artificially recruiting PTB to either side of FGF-R2 exon IIIb resulted in the repression of exon IIIb (Appendix IV. Fig 2). These experiments are more thoroughly described in the manuscript entitled "RNAi Mediated PTB Depletion Leads to Enhanced Exon Definition" (Appendix IV), which has been revised and resubmitted to Cell/Molecular Cell and is awaiting editorial review. The experiments described here and in the first annual report we feel sufficiently address the goals outlined in task one of the Statement of Work.

Task 2 in the statement of work is aimed at demonstrating that endogenous PTB is the actually involved in the repression of exon inclusion. In order to address this task it was necessary to develop RNAi as a protocol in order to deplete endogenous PTB. It has been recently demonstrated that the transfection of short RNA duplexes specific to target gene mRNA can result in the specific, yet transient, destruction of that RNA thus resulting in eventual *in vivo* protein depletion (Elbashir et al, 2001). Several siRNA duplexes were designed against PTB and tested in human 293T and HeLa cells (Fig 3a). Transfection of one of these duplexes (PTB 9) was found to result in dramatic depletion of endogenous PTB (Fig 3b). The analogous version of

this duplex was used to knockout PTB in rat DT3 cells and found to increase FGF-R2 exon IIIb inclusion in rat derived FGF-R2 minigenes (Appendix IV. Fig 3). The most important discovery summarized in this report and potentially in this entire funded research initiative is that RNAi mediated depletion of endogenous PTB led to an increase in FGF-R2 exon IIIb in endogenous mRNA transcripts (Appendix IV. Fig 4). Furthermore, depletion of endogenous PTB led to an increase in the inclusion of fibronectin EIIIb exon, which is another exon suspected to be repressed by PTB. These data are important in that it is the first demonstration of endogenous PTB function on endogenous alternative splicing. It is also, to the best of our knowledge, the first demonstration of the function of an alternative splicing factor using RNAi.

One other achievement that was spurred by a collaborative effort with Dr. Yan Zheng of Dr. Bryan Cullen's laboratory at Duke was the use of DNA plasmids to elicit RNAi mediated depletion of endogenous PTB. This data is thoroughly described in the appended reprint (Appendix V) entitled "Both Natural and Designed Micro RNAs can inhibit the expression of Cognate mRNAs when expressed in Human Cells". We view this manuscript as a by-product of achieving task 2a, which was to develop RNAi technology to deplete endogenous PTB.

The final aim of task 2 was to test the fluorescent reporters designed to report PTB mediated silencing *in vivo* for their sensitivity to RNAi-mediated PTB depletion. This experiment was performed once and failed due to a technical flaw in the protocol. Specifically, neomycin used to maintain stable DT3 cells containing the fluorescent reporter must be removed to perform the RNAi technique. This was not known *a priori* and will be re-attempted in the coming months.

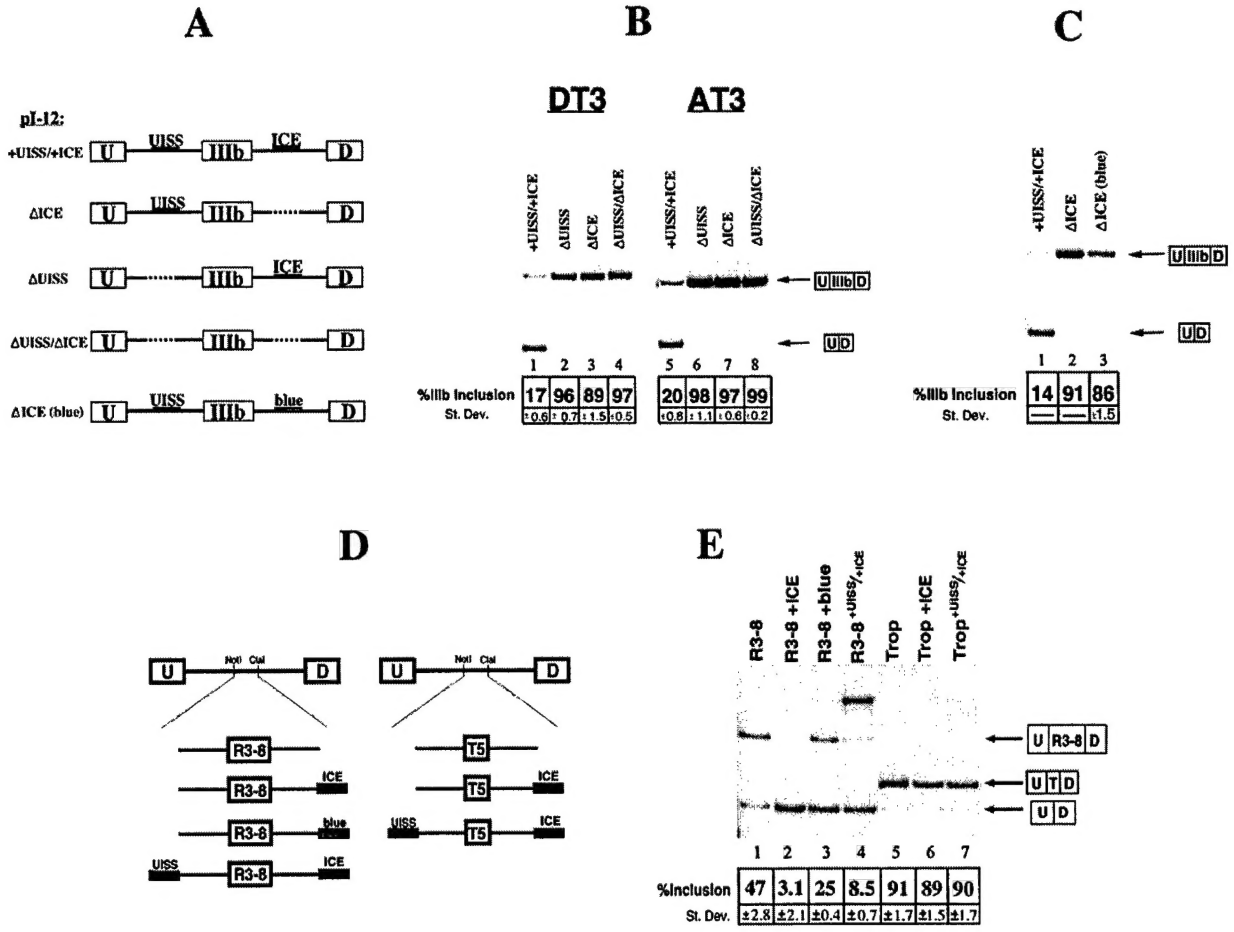

Figure 1. The ICE is required to repress FGF-R2 exon IIIb and is sufficient to repress FGF-R3 exon 8. A. The design of five minigenes used in the following two panels. Various deletions, as indicated by dashes, were made involving the Upstream Intronic Splicing Silencer (UISS) or the Intronic Control Element (ICE). The minigenes were used for transfection into both DT3 and AT3 cells and assayed by RT-PCR using minigene specific primers. **B.** RT-PCR analysis of the transfection of minigenes testing the silencing activity of the ICE on exon IIIb. The expected products of exon IIIb inclusion and skipping are denoted on the right and the percentage of exon IIIb inclusion is calculated using the molar equivalents of each product in this equation: (U-IIIb-D)/(U-IIIb-D + U-D). Averages and standard deviations are the result of triplicate stable transfections. **C.** The result of substituting an irrelevant size matched bluescript sequence in place of the ICE. This transfection was performed in DT3 cells; however, AT3 cells yielded similar results (not shown). **D.** The ICE and/or UISS were inserted in flanking regions of FGF-R3 exon 8 (R3-8) or cardiac Troponin T exon 5 (T5). The bluescript insertion was the same as used in FGF-R2 **E.** The results of transfection of heterologous constructs into AT3 cells. Splicing products are labeled accordingly. The unlabeled product in lane 4 is a splice product resulting from the use of a cryptic 3' splice site within the UISS. Transfections into DT3 cells yielded similar results.

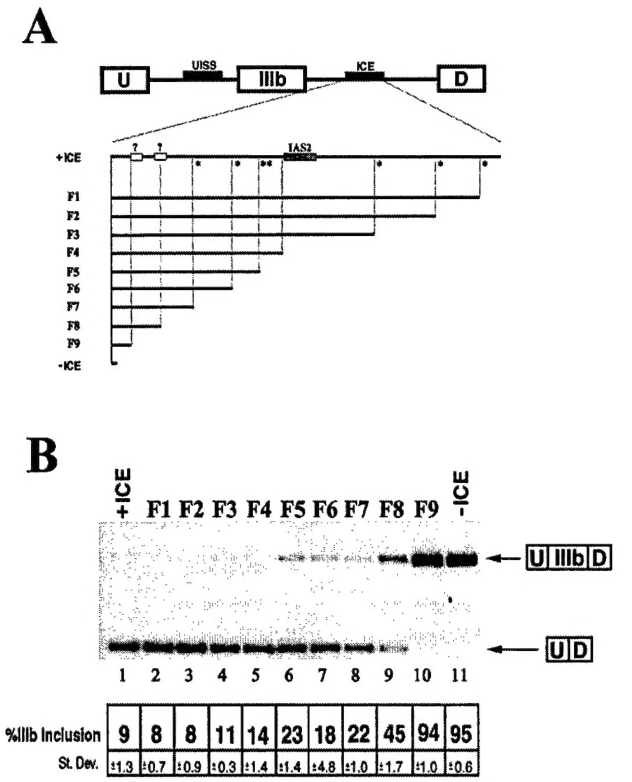

Figure 2. Deletion from 3' to 5' dissects the intronic control element. A. This panel depicts 3' to 5' deletion constructs labeled F1 through F9. Consensus PTB binding sites are noted by the asterisks, IAS2 is labeled as such, and the highly conserved sequences at the 5' end of the ICE are shown as boxes with "?" above them. **B.** In this panel are the results of RT-PCR analysis of stable transfections of these constructs into DT3 cells. Quantifications are representative of triplicate stable transfections.

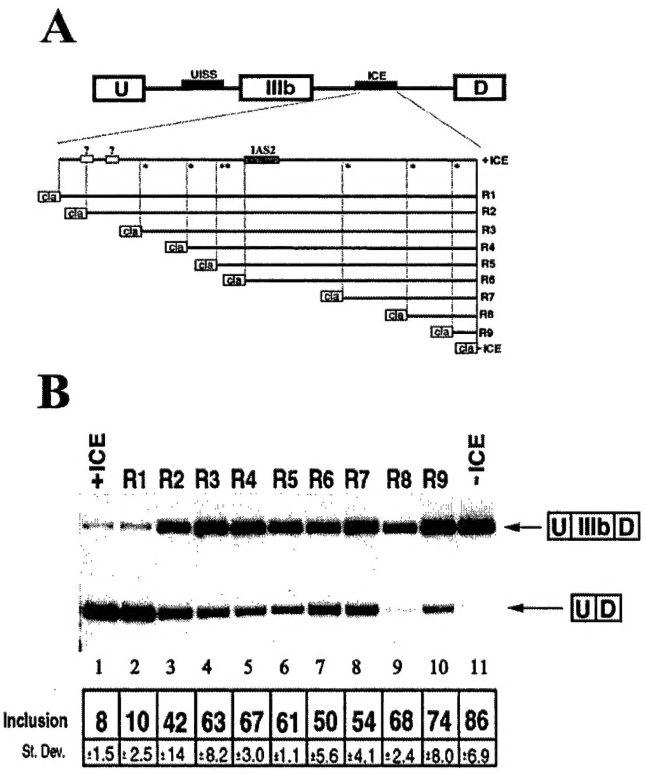

Figure 3. Deletion from 5' to 3' dissects the intronic control element. A. The upper panel depicts 5' to 3' deletion constructs labeled R1 to R9. Each construct contains an addition of a ClaI restriction site at the 5'end. **B.** In the bottom panel are the results RT-PCR analyses of stable transfection of these constructs into DT3 cells. Quantifications are again the results of triplicate stable transfections. Similar results were attained when all constructs were transfected into AT3 cells (not shown).

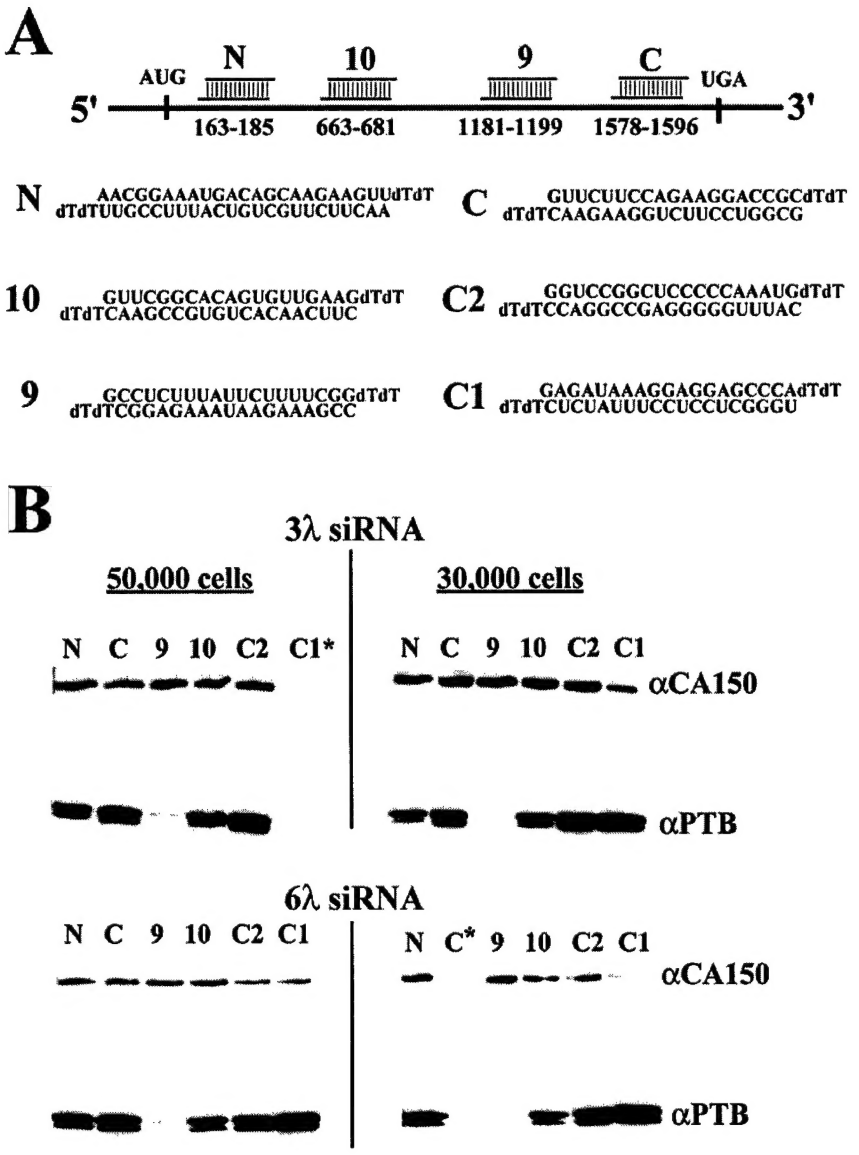

Figure 4. PTB siRNA duplexes possess different potentials for RNA interference. A. Four siRNA duplexes were designed to target the PTB mRNA using the rule of AA-N19. The sequence and the relative position of the target within the mRNA are depicted. The sequence of the positive control siRNA duplex (C1), which has been shown to deplete levels of CA150 is shown as well as the negative control C2 duplex, which has been found not to decrease CA150 levels. **B.** The upper two western blots are the results of transfection of 3 ul of the depicted siRNA duplexes in 293T cells at two different plating densities. The lower two blots represent results from an identical experiment with the exception that 6 ul of each siRNA duplex was used. The lanes depicted with a "*" represent skipped lanes due to experimental error.

Key Research Accomplishments

-Identified regions of PTB binding downstream of exon IIIb and defined them as splicing silencers.
-Recruited PTB via heterologous MS2 fusion demonstrating PTB's sufficiency in repression
-Developed RNA interference protocol to transiently knock out PTB
-Demonstrated the function of endogenous PTB to repress endogenous FGFR2 IIIb inclusion using RNAi

Reportable Outcomes

-**Manuscript:** **Zeng Y.,** ***Wagner EJ,*** **Cullen BR.** 2002 Both Natural and Designed Micro RNAs can inhibit the expression of Cognate mRNAs when expressed in Human Cells. Mol Cell. **9**: 1327-1333 (see appendix)

-**Manuscript:** ***Wagner EJ*** **& Garcia-Blanco MA.** 2002 RNA Interference Demonstrates PTB as an Antagonist of Exon Definition (manuscript resubmitted to Cell; see appendix)

-**Manuscript:** ***Wagner EJ,*** **Curtis M, Eis P, & Garcia-Blanco MA.** 2002 The Use of RNA Invader to Assay to Measure FGF-R2 Alternative Splicing Events. (man in prep)

-**Manuscript:** ***Wagner EJ,*** **& Garcia-Blanco MA.** 2001 The Polypyrimidine Tract Binding Protein Antagonizes Exon Definition. Mol. Cell. Biol. **21:** 3281-3288

-**Manuscript:** **Carstens RP*,** ***Wagner EJ*,*** **& Garcia-Blanco MA.** 2000. An Intronic Splicing Silencer Causes Skipping of the IIIb Exon of Fibroblast Growth Factor Receptor 2 through Involvement of Polypyrimidine Tract Binding Protein. Mol. Cell Bio. **20**: 7388-7400 **(*co-first authors).** Please Note: This work was performed just prior to funding period but was Published during funding period. The work presented in this paper Is consistent with numerous aspects of the proposed research.

-**Presentation:** Oral Presentation at the Annual RNA Society Meeting held in Madison Wisconsin. Title of Abstract: Cell-type specific Inclusion of FGF-R2 exon IIIb is mediated by a central switch: the intronic control element. (See appendix)

-**Presentation:** Poster Presentation at the Annual RNA Society Meeting held in Madison Wisconsin. Title of Abstract: Characterizing Splicing Repressors of FGFR2 exon IIIb (See Appendix)

-**Degrees:** Received Doctor of Philosophy from the Program in Molecular Cancer Biology at Duke University. Title of Thesis: The Polypyrimidine Tract Binding Protein Antagonizes Exon Definition

Conclusions

Since the sequencing of the human genome it has become clear that alternative splicing is more of a rule than an exception. Estimates suggest that anywhere from 40% to 70% of genes undergo alternative splicing. As important as this process is, so little is known about the proteins involved and especially about their molecular mechanisms. The achievements in this report are significant in the fact that they provide strong evidence for the endogenous function of one of the regulators of alternative splicing; PTB. The alternative splicing of multiple genes have been found to change as breast cancer cells form and undergo tumor progression (as well as many other cancers) thus the function of the proteins governing this process is likely changing. Identifying and characterizing these proteins is integral to predict changes in splicing patterns. The conclusions of this midterm report are straightforward: the function of endogenous PTB is to antagonize the exon definition of FGF-R2 exon IIIb, fibronectin EIIIb, and most likely exons form other genes. The final year of funding will be important because it is aimed at determining how cells usurp this repression mediated by PTB.

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Appendix

I. Amended Statement of Work (Pending)

Revised Statement of Work
DAMD-17-00-1-0236

The Involvement of the Polypyrimidine Tract Binding Protein (PTB) in the Alternative Splicing of Fibroblast Growth Factor Receptor 2 (FGF-R2)

Task 1. Identify PTB binding sites within FGF-R2 intronic regions and develop fluorescent alternative splicing reporters to recapitulate the *in vivo* alternative splicing of FGF-R2 (Months 1-12)

a. Map PTB binding sites within intronic regions flanking IIIb using UV crosslinking and RNA gel shift assays. (Months 1-3)
b. Determine functional relevance of PTB binding sites using minigene assays (Months 4-6)
c. Develop a series of fluorescent reporters using either GFP or RFP capable of recapitulating PTB repression of FGF-R2 exon IIIb (Months 7-12)

Task 2. Use fluorescent reporters as well as standard minigene constructs to determine the role of PTB in the repression of FGF-R2 exon IIIb (Months 13-24)

a. Develop RNA interference (RNAi) protocol to transiently knock-out PTB (Months 13-18)
b. Examining both endogenous FGF-R2 mRNA and FGF-R2 minigenes, apply RNAi technology to determine the involvement of endogenous PTB in the repression of exon IIIb (Months 19-21)
c. Test the effects of RNAi to PTB on the fluorescent splicing reporters to determine Their sensitivity to *in vivo* PTB levels (Months 22-24)

Task 3. Define cell-type specific mechanism causing the derepression of PTB (Months 25-36)

a. Using both deletion and point mutagenesis of minigenes, define cell-type specific activating elements capable of activating IIIb inclusion (Months 25-30)
b. Determine mechanism of PTB derepression via cell-type specific activation (Months 26-30)

II. Abstract for Oral Presentation at Annual RNA Conference held in Madison WI May 2002.

Quantitative Study of Alternative Splicing for the Fibroblast Growth Factor Receptor 2 Using the Invader RNA Assay

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The ability to precisely differentiate between mRNA isoforms is critical to the study of alternative splicing. Many currently used detection methods suffer from difficulty, high cost, lack of reproducibility, insufficient quantification, and most important, lack of specificity. The Invader RNA assay, which specifically and quantitatively measures zeptomole levels of RNA, appeared to be ideal for the quantification of alternative mRNAs. We applied this technology to investigate the alternative inclusion of exon IIIb or exon IIIc in mRNAs encoding Fibroblast Growth Factor Receptor 2 (FGF-R2) in two related cell lines derived from the rat Dunning prostate tumor, DT3 and AT3. DT3 cells used exon IIIb and AT3 included exon IIIc. IIIc inclusion in AT3 cells requires the silencing of IIIb and is driven by strong splice sites bordering the IIIc exon. IIIb inclusion in DT3 cells requires two regulatory elements, ISAR and IAS2, which jointly activate exon IIIb and repress exon IIIc. In this study, we transfected several FGF-R2 minigenes containing various deletions of IAS2 and ISAR into DT3 and AT3 cells and used the Invader assay to distinguish and quantify the splicing products. The IIIb and IIIc Invader probe sets were highly specific for their respective targets (i.e., no cross-reactivity). Further, we compared Invader assay results with RT-PCR results. Whereas both methods yielded similar splicing profiles, only the Invader assay precisely quantified small variations in the absolute number of spliced products. This study indicates the Invader RNA assay is a highly specific and quantitative alternative to current detection methods.

III. Abstract for Poster Presentation at Annual RNA Conference held in Madison WI May 2002.

Characterizing Splicing Repressors or FGF-R2 Exon IIIb

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The tissue specific regulation of fibroblast growth factor receptor 2 (FGF-R2) pre-mRNA splicing results in the mutually exclusive use of either the IIIb exon or the IIIc exon. Choice of either of these exons results in receptors with differing ligand binding properties. To study this regulation, we use the well-differentiated DT3 rat prostate carcinoma cell line, which includes only the IIIb exon, while the poorly differentiated AT3 rat prostate carcinoma cell line includes exon IIIc.

Downstream of exon IIIb lies an Intronic Control Element (ICE) which elicits multifunctional regulation on both exon IIIb and IIIc. One of these functions is to silence exon IIIb. Within the ICE are 7 UCUU motifs, which are required for efficient silencing of exon IIIb, and that bind PTB. A critical role for PTB is supported by the following: We have previously shown that overexpression of PTB enhances the silencing of IIIb and now we show that recruitment of a PTB-MS2 fusion protein to a heterologous binding site within the ICE leads to silencing of IIIb. We also show that specific depletion of PTB in vivo using RNA interference leads to an increase in exon IIIb inclusion. We have found that minigenes with several UCUU elements deleted are predictably more sensitive to the levels of PTB in the cell. The increase in exon IIIb inclusion can be partially rescued by overexpression of human PTB, which is encoded by a mRNA expected to be partially resistant to RNAi.

A second silencing element has been found at the 5' end of the ICE, which we believe does not bind PTB. This sequence is highly conserved among FGF-R2 genes from rat to sea urchin. We are using single point mutations that destroy silencing to design a purification scheme to identify this factor(s). We will discuss the progress of this purification.

IV. Manuscript re-submitted and under editorial review at Cell

RNAi Mediated PTB Depletion Leads to Enhanced Exon Definition

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Running Title: PTB antagonizes exon definition

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Summary

Mutually exclusive use of exons IIIb or IIIc in fibroblast growth factor receptor-2 transcripts requires the silencing of exon IIIb. This silencing is mediated by an intronic splicing silencer upstream of the exon and by an intronic control element downstream. Both of these elements bind the polypyrimidine tract binding protein (PTB) and PTB binding sites within these intronic elements are required for efficient silencing of exon IIIb. Recruitment of an MS2-PTB fusion protein to sequences upstream or downstream of exon IIIb causes repression of this exon. Depletion of endogenous PTB using RNAi results in an increase in IIIb inclusion in transcripts derived from a minigene constructs and from the endogenous FGF-R2 gene. This is the first use of RNAi technology to demonstrate the *in vivo* function of a splicing factor. Most importantly, these data demonstrate that PTB is a negative regulator of exon definition.

Introduction:

The more than twenty-five fibroblast growth factors (FGFs) mediate their action via four well-characterized plasma membrane receptors (FGF-Rs), which contain a C-terminal bipartite tyrosine kinase domain and an extracellular FGF binding domain composed of two or three immunoglobulin-like Ig domains. The two C-terminal Ig domains, Ig-II and Ig-III, determine ligand binding specificity (Miki et al., 1992). Three of the FGF-Rs, FGF-R1, FGF-R2 and FGF-R3, exist as multiple isoforms that have one of two different Ig-III domains and thus show isoform-specific affinity for FGFs. This receptor diversity is the result of alternative splicing of FGF-R primary transcripts. The gene structure of FGF-R2 reveals that the N-terminal half of Ig-III is encoded by exon IIIa in both isoforms, whereas the C-terminal half is encoded by either exon IIIb in the FGF-R2 (IIIb) isoform or exon IIIc in the FGF-R2 (IIIc) isoform. The mutually exclusive use of exon IIIb or exon IIIc is tightly regulated such that FGF-R2 (IIIb) is the predominant, if not sole, isoform of FGF-R2 in epithelial cells, and FGF-R2 (IIIc) is the isoform of choice in mesenchyme (De Moerlooze et al., 2000). Proper isoform expression is necessary for the FGF/FGF-R2 signaling that mediates mesenchymal-epithelial interactions, which are required for organogenesis in both mouse and human embryos (De Moerlooze et al., 2000; Hajihosseini et al., 2001; Oldridge et al., 1999). Inappropriate expression of FGF-R2 (IIIc) during progression of prostate carcinomas suggests that abnormal regulation of FGF-R2 isoform choice accompanies tumorogenesis (Matsubara et al., 1998).

The mutually exclusive incorporation of exon IIIb or exon IIIc in FGF-R2 mRNAs is regulated by cis-acting elements in the FGF-R2 pre-mRNA and by trans-acting factors, which can be ubiquitous or cell-type specific. Using the well differentiated rat prostate carcinoma DT3 line, which expresses FGF-R2 (IIIb), and the poorly differentiated AT3 line, which expresses

FGF-R2 (IIIc), we identified an upstream intronic splicing silencer (UISS). UISS, which is located 50 nucleotides away from of exon IIIb, is required for skipping of exon IIIb in AT3 cells (Carstens et al., 2000). This skipping is facilitated by an exonic splicing silencer (ESS), and by weak splice sites flanking exon IIIb, which were originally identified in the human exon IIIb (Del Gatto and Breathnach, 1995; Del Gatto et al., 1996). Immediately downstream of exon IIIb lie several activating elements, IAS1, IAS2 and ISAR (or IAS3) (Carstens et al., 1998; Del Gatto et al., 1997), which in epithelial cells counter the inherent weakness of exon IIIb and the repressive action of the intronic silencer. In summary, the FGF-R2 isoform choice is set up by the global silencing of exon IIIb, countered by a combination of cell-type specific activation of exon IIIb and repression of exon IIIc.

We have previously shown that UISS is a bipartite silencer of exon IIIb with each of its two components contributing equally to IIIb silencing. The 5' component of UISS, or ISS1, resembles previously described pyrimidine-rich silencers, whereas the 3' component, ISS2, contains UG repeats. ISS1 binds the polypyrimidine tract binding protein (PTB) and this binding correlates with silencing of exon IIIb (Carstens et al., 2000). Overexpression of PTB leads to profound silencing of minigene constructs that have an intact ISS1 and if ISS1 is mutated, such that it can longer bind PTB, the effect on exon IIIb inclusion is significantly lower (Carstens et al., 2000). PTB was discovered as a protein in HeLa cell nuclear extracts that bound U-rich polypyrimidine tracts in introns (Garcia-Blanco et al., 1989). Although the binding to intronic polypyrimidine tracts and biochemical complementation assays (Gil et al., 1991; Patton et al., 1991) originally suggested a possible role for PTB in constitutive splicing, Helfman and colleagues showed that PTB binding sites are required for skipping of exon 7 in ß-tropomyosin transcripts (Mulligan et al., 1992). These authors were the first to propose that PTB functions as

a repressor of exon inclusion, a view subsequently supported by much data and formally proven in this manuscript. In addition to the repression of exon 7 of ß-tropomyosin and exon IIIb in FGF-R2, PTB has been implicated in the silencing exon 24 of $GAB_A\gamma 2$ (Zhang et al., 1999), exon N1 of c-src (Chan and Black, 1997), exon SM of α-actinin (Southby et al., 1999), exon EIIIB in fibronectin (Norton, 1994), exon 9 of caspase-2 (Cote et al., 2001), the α exon of FGF-R1 (Jin et al., 2000), exon 3 of α-tropomyosin (Gooding et al., 1998), and more recently exon IIIc of human FGF-R2 (Le Guiner et al., 2001). The *in vitro* depletion of PTB results in increased exon inclusion and re-addition of purified or recombinant PTB restores higher levels of exon skipping (Chou et al., 2000; Southby et al., 1999). Although carefully controlled, these experiments are open to the criticism that the results are *in vitro* observations that require confirmation *in vivo*. Overexpression of wild-type PTB in cells in culture has led us and others to conclude that this protein can silence exons *in vivo* (Carstens et al., 2000; Wollerton et al., 2001; Liu et al., 2002). Recently, Charlet et a. (2002) showed that overexpression of a truncated form of PTB enhances the inclusion of the cardiac troponin T exon 5 from a minigene construct and suggested that this truncated protein behaved as a dominant negative PTB. Given the uncertainties associated with the overexpression of the truncated PTB even this experiment did not prove that endogenous PTB has an exon silencing role in cells. This proof awaited experiments that would deplete PTB *in vivo*, as shown below.

Here we describe the identification of an element that lies 79 nucleotides downstream of exon IIIb and which we named the intronic control element (ICE). ICE contains seven PTB binding sites, which are required for full silencing activity. We show that artificial recruitment of PTB-MS2 coat protein chimeras to UISS and ICE via MS2 stemloop RNA sites leads to silencing of exon IIIb. These data led us to conclude that PTB can silence exon IIIb via ISS and

ICE and raised the question: is this a physiological role of PTB? To answer this question we depleted endogenous PTB using RNA interference (RNAi) and showed that PTB depletion led to increased exon IIIb inclusion in transcripts from a minigene and from the endogenous FGF-R2 gene. These experiments prove that PTB antagonizes exon IIIb definition in FGF-R2 transcripts.

Results:

Exon IIIb is flanked by intronic silencers that bind PTB. Phylogenetic comparison of human, rat, mouse, and chicken FGF-R2 sequence data led to the recognition of a conserved region 79 nts. downstream of exon IIIb. Although the center of this region contains the intronic activating sequence 2 (IAS2), which in epithelial cells is involved in the activation of exon IIIb (Del Gatto et al., 1997), the function of the larger conserved region was unknown. To characterize the function of this region, which spans 239 nts., we transfected FGF-R2 minigenes with the sequence either deleted or replaced with irrelevant sequences and noted that disruption of this element caused derepression of exon IIIb in both DT3 and AT3 cells (data not shown). Because this region was involved in both exon inclusion and skipping we termed this the intronic control element (ICE). Using RT-PCR we also showed that placement of ICE downstream of exon 8 of FGF-R3 led to repression of that exon, whereas equal placement of this element downstream of exon 5 of the cardiac troponin gene did not have any effect (data not shown). This suggests that the ICE is sufficient to silence a subset of heterologous exons, possibly those with weak 5' splice sites. These experiments and previous data (Carstens et al., 2000) reveal that exon IIIb is flanked by silencers, UISS upstream and ICE downstream, that repress its inclusion.

We used extensive mutational analysis to assess the importance of different regions within ICE and concluded that the potent silencing activity of ICE is mediated by two sub-elements: the downstream intronic splicing silencer 1 (DISS1) containing four UCUU motifs and a highly

conserved compact element at the 5' end (5'CE), which will not be discussed further, and the DISS2, which contained three more UCUU motifs (Wagner et al., unpublished results). In total, we identified seven UCUU sequences, which represent putative binding sites for PTB (Perez et al., 1997; Singh et al., 1995) (* in Fig. 1A). UV crosslinking analysis confirmed that the UCUU-rich sequences indeed bound PTB *in vitro* (Fig. 1B). Previously, we had shown that PTB bound to the UISS1 sub-element of UISS upstream of exon IIIb (Carstens et al., 2000). To compare the relative contributions of the PTB binding sites located in UISS1 and those within ICE, we constructed minigenes deleted in sequences found to crosslink specifically to PTB (Fig. 1C). Results, shown in Figure 1D, reveal that PTB binding sites within the ICE make a greater contribution to exon IIIb silencing than the PTB binding sites within UISS1. It is important to note that ISAR has been deleted in all minigenes used in this report and therefore silencing of exon IIIb is dominant even in DT3 cells. The data above provide a correlation between PTB binding *in vitro* and exon IIIb silencing *in vivo* and suggest a topology where exon IIIb is flanked by PTB binding sites, which collaborate to silence exon definition.

Heterologous Recruitment of PTB to UISS1 and ICE results in exon IIIb silencing. We sought to determine if the binding of PTB was sufficient to elicit exon IIIb silencing. We inserted four copies of the MS2 RNA stem loop (Wiegand et al., 2002) in place of the PTB binding sites within UISS1 and ICE (Fig. 2A). When a minigene containing PTB binding sites substituted with MS2 RNA both upstream and downstream of exon IIIb (MS2 Up/Dn) was co-transfected with a construct that overexpressed MS2-Tap or HisG-PTB proteins there was no effect on exon IIIb inclusion, however, when co-transfected with a construct that overexpressed an MS2-PTB fusion protein we found a dose-dependent repression in exon IIIb inclusion (Fig. 2B). This repression reached about a three-fold reduction in exon IIIb inclusion. It was not clear

from these data if recruitment of PTB to either side of exon IIIb alone was sufficient to elicit exon IIIb silencing. To address this question MS2 RNA binding sites were substituted for the endogenous PTB binding sites either upstream (MS2 Up) or downstream (MS2 Dn) of exon IIIb (Fig. 2A). Minigenes were co-transfected with the MS2 vector alone or the MS2-PTB fusion. As expected a minigene that lacked the MS2 RNA sequence (ΔMS2) was unresponsive to the expression of the MS2-PTB fusion protein thus demonstrating the requirement for the MS2 RNA sequence for MS2-PTB repression (Fig. 2C). MS2-PTB recruitment upstream of exon IIIb led to a slight reduction in IIIb inclusion, recruitment downstream of exon IIIb resulted in a greater amount of repression, and recruitment upstream and downstream led to more potent repression (exon IIIb inclusion diminished from 16% to 5.3%; Fig. 2C). These results show that when PTB is artificially recruited to intronic sequences upstream or downstream of exon IIIb, it is capable of repressing exon IIIb.

***In vivo* knockdown of PTB by RNA interference increases exon IIIb inclusion.** Technology has recently become available to transiently knockdown levels of exogenous or endogenous proteins within mammalian tissue culture systems based on the use of RNA interference (RNAi) (Elbashir et al., 2001; reviewed in Hannon, 2002). A short interfering RNA (siRNA) was designed against the rat PTB mRNA (termed "PTB" in relevant panels of Fig. 3). Treatment of DT3 cells with the PTB siRNA, but not with a non-specific siRNA, resulted in a transient reduction of endogenous PTB levels, while not affecting the unrelated CA150 protein (Fig. 3A and data not shown). PTB knockdown had only a minimal effect on exon IIIc inclusion, which had previously been noted to be repressed only by exceedingly high levels of PTB overexpression (Le Guiner et al., 2001) (Fig. 3B). siRNA-mediated knockdown of PTB, however, led to more than a three-fold increase in exon IIIb inclusion (Fig. 3B). Thus, these data

suggest that endogenous PTB functions to antagonize exon IIIb definition *in vivo* in the context of minigene constructs.

To address the effect of PTB depletion on the inclusion of exon IIIb among endogenous FGF-R2 transcripts we used 293T cells, which normally include exon IIIc in their endogenous FGF-R2 mRNA (Yeakley et al., 2002). In order to target the human PTB mRNA we designed an siRNA that would target this mRNA in an analogous position to the rat PTB mRNA. Transfection of the PTB siRNA into 293T cells led to a profound and lasting knockdown of PTB levels (Fig. 4A). Concomitant with the depletion of PTB we observed an accumulation of endogenous FGF-R2 transcripts containing exon IIIb (Fig. 4B), which was observed as an increase in the percentage of transcripts including both IIIb and IIIc exons (double inclusion). The percentage of double inclusion increased from 7.4% before treatment to 23% by 120 hours after siRNA transfection. Inclusion of other exons thought to be repressed by PTB was also expected to increase. Inclusion of one such exon, exon EIIIB of fibronectin mRNAs (Norton, 1994), was increased from 7.2% to 42% at 216 hours post initial siRNA transfection (Fig. 4C). These data indicate a physiological role for PTB in silencing exon IIIb of FGF-R2 and exon EIIIB of fibronectin, and suggest a general role for PTB in silencing regulated exons.

Discussion:

PTB can antagonize exon IIIb inclusion. All forms of alternative splicing can be viewed as alternative modes of exon definition (Berget, 1995; Robberson et al., 1990). Thus splicing enhancers can be seen as elements that enhance the definition of an exon and splicing silencers as those that antagonize exon definition (Wagner and Garcia-Blanco, 2001). Exon IIIb definition can be significantly decreased by overexpressing PTB, regardless of which isoform, and this

effect is dependent on PTB binding sites within UISS (Carstens et al., 2000). These findings and the discovery of potential PTB binding sites downstream of exon IIIb led us to propose that multiple PTB molecules bind upstream and downstream of exon IIIb creating a "zone of silencing" (Wagner and Garcia-Blanco, 2001). We also proposed that given the ubiquitous presence of PTB, silencing would be potentially found in most cells, which implies that cell-type specific inclusion of exon IIIb is mediated by specific countermeasures that activate exon IIIb. Several aspects of this proposal were confirmed by experiments presented in this manuscript.

Here we show that UISS and ICE work in concert to repress exon IIIb inclusion. We propose that these two intronic silencers conspire with the hnRNP A1 binding ESS (Del Gatto-Konczak et al., 1999) and the constitutively weak splice sites that define exon IIIb (Del Gatto and Breathnach, 1995) to suppress the inclusion of this exon in non-epithelial cells. Although epithelial cells include exon IIIb in FGF-R2 mRNAs, silencing of exon IIIb by UISS and ICE can be observed in these cells with minigene constructs missing the ISAR element (Carstens et al., 2000)(Fig. 1). The unmasking of the silencing activity in epithelial cells indicates that UISS and ICE mediate their action via factors that are present in both epithelial and mesenchymal cell types. Indeed, one such factor, PTB was known to be present in both DT3 and AT3 cells (Wagner et al., 1999) and had been implicated in mediating UISS function (Carstens et al., 2000). Here we demonstrated that PTB bound two regions (DISS1 and DISS2) within ICE and that these regions were important for silencing exon IIIb (Fig. 1). Deletion of the PTB binding sites within ICE resulted in approximately three-fold enhancement in exon IIIb inclusion vs. a two-fold enhancement when PTB binding sites in UISS were deleted, suggesting that the overall impact of PTB on ICE action was more important. The importance of PTB binding to ICE was also noted when we showed that PTB can silence exon IIIb if artificially recruited to ICE via a

bacteriophage MS2 coat protein RNA binding site (Fig. 2). Recruitment of an MS2-PTB fusion protein to both UISS and ICE, however, is much more effective than recruitment to either upstream or downstream element alone.

The data summarized above are consistent with many observations of others showing that PTB binding sites within alternative splicing substrates were necessary for silencing and with a handful of observations showing that overexpression of PTB can silence an alternative exon (Carstens et al., 2000; Wollerton et al., 2001; Liu et al., 2002). Taken together, the *in vivo* data summarized above indicate that PTB can antagonize exon inclusion. This conclusion begs the question; does endogenous PTB carry out this function in cells?

PTB antagonizes exon IIIb definition. The function of PTB in nuclear extracts has been addressed by depleting PTB, either using anti-PTB antibodies (Chou et al., 2000) or RNA affinity matrices (Southby et al., 1999). PTB depletion was shown to enhance inclusion of several regulated exons and readdition of recombinant PTB restored diminished levels of exon inclusion (Chou et al., 2000; Southby et al., 1999). It has been recently found that overexpression of a truncated form of PTB, which presumably acts as a dominant negative, enhances the inclusion of the normally repressed cardiac troponin T exon 5 (Charlet et al., 2002). Although this experiment suggests a silencing role in vivo, it is not clear how the truncated form of PTB behaves in a dominant-negative fashion and we have not found the same effect on FGF-R2 alternative splicing when overexpressing this truncated PTB (Wagner et al., unpublished results).

Although the aforementioned experiments were carefully performed, definitive evidence for the involvement of PTB in exon silencing required the depletion of PTB in living cells. To this

end we have used a modification of the RNAi method of Elbashir et al (2001) to specifically knockdown PTB to almost undetectable levels in DT3 and 293T cells (Fig. 3 and 4). The knockdown of PTB resulted in a two to three fold increase in the ratio of inclusion of IIIb to skipping in minigenes, which was the expected effect given our data with the deletion of the PTB binding sites and the artificial recruitment of MS2-PTB to the transcripts. Most importantly, the depletion of PTB from 293T cells led to an accumulation of endogenous spliced products containing both exons IIIb and IIIc (Fig. 4). The apparent three-fold increase in these transcripts very likely under represents the effect of PTB depletion because transcripts containing both exons IIIb and IIIc are subject to nonsense-mediated decay (Jones et al., 2001). Not surprisingly the knockdown of PTB also affected the splicing pattern of endogenous fibronectin transcripts, which have multiple PTB binding sites flanking the EIIIB exon (Norton, 1994). Inclusion of EIIIB increased six fold in response to PTB depletion (Fig 4). We conclude from these experiments that PTB antagonizes the definition of FGF-R2 exon IIIb and fibronectin EIIIB exon and believe that PTB plays a similar silencing role for other regulated exons. To the best of our knowledge this is the first use of RNAi to investigate the role of a splicing factor.

Dispensing with elegance: combinatorial solutions. Deletion of PTB binding sites and knockdown of PTB both led to an approximately three fold increase in exon IIIb inclusion (Fig. 1), whereas deletion of a complete ICE led to a ten fold increase in exon inclusion (data not shown). The most likely explanation for these results is that PTB collaborates with other unidentified factors, which bind to UISS2 and to 5' CE. The need for multiple factors to bind adjacent elements to integrate an alternative splicing outcome has been noted in several cases. HnRNP H, hnRNP F, KSRP and nPTB have been found to bind the downstream splicing

enhancer, which is required for inclusion of the N1 in c-src mRNAs in neural tissues (Chou et al., 1999; Markovtsov et al., 2000; Min et al., 1995; Min et al., 1997). The tissue specific inclusion of the alternative exon 5 of cardiac troponin-T appears to require the integrated activity of PTB and members of the msl family of factors (Charlet et al., 2002). PTB associates with FBP and Sam68 on the intron upstream of the regulated exon 7 in rat β-tropomyosin transcripts (Grossman et al., 1998). The need to regulate a vast number of alternative splicing events has been solved by the integration of the activity of a limited number of factors that by combinatorial assortment can lead to very large number of functional states (Smith and Valcarcel, 2000). An elegant binary switch provided by a single alternative splicing factor, as is the case for Sxl protein in *Drosophila melanogaster*, may be reserved for crucial decisions, such as sex determination, which are made very early in development.

The cell-type specific activation of FGF-R2 exon IIIb. The mutually exclusive use of exon IIIb in epithelial cells (DT3) and exon IIIc in fibroblasts and de-differentiated prostate carcinoma cells (AT3) is tightly regulated. It is clear from data presented here and our prior results that the factors that mediate silencing of exon IIIb via UISS and ICE are present and active in both cell-types. How, then, is exon IIIb included in FGF-R2 mRNAs in DT3 cells? Several possibilities, which are not exclusive of each other, can be envisioned. We have previously shown that the predominant PTB isoform is different in AT3 and DT3 cells, and therefore, although all PTB isoforms appear to equally silence exon IIIb (Carstens et al., 2000), one could posit that the PTB4 isoform that predominates in DT3 cells can be countered more readily by factors recruited via the IAS2 and ISAR elements. Even though this difference in PTB isoform expression may contribute to cell-type specific inclusion of exon IIIb, it is likely that an epithelial specific factor or perhaps a combination of factors results in the specific de-repression of exon IIIb. By

extrapolation from the insightful observations made for N1 exon inclusion in c-src (Chou et al., 2000), we suggest that cell-type specific activators displace PTB and possibly the factors that interact with UISS2 and 5' CE. Our data and that of Breathnach and McKeehan indicate that the IIIb activating factors are recruited via IAS2 and ISAR, which work in concert and form a secondary structure that is required for their function (Del Gatto et al., 1997; Jones et al., 2001). Given that IAS2 is embedded within ICE, it is reasonable to predict that the IAS2-ISAR structure, which should be stabilized by the cell-type specific activating factors, will disrupt the silencing topology. Thus the regulation of exon IIIb inclusion in epithelial cells is mediated by countering the default repression mechanism instituted by PTB and other unknown splicing repressors.

Experimental Procedures

Minigene designs. The pI-12 construct was previously described (Carstens et al., 1998). Construction of the ICE containing minigenes were using the upstream primer Int3BF2 (Carstens et al., 2000) and the reverse primer Int3BR3cla (5'GGCCATCGATAGAAGTGTGCA GTCCAAACAAG 3') whereas the ICE deletion used the reverse primer Int3BR2cla (5' GGCCA TCGATCTCCTATATTCAGTTTTCTTAA 3'). MS2 RNA sequences were cloned using PCR products were constructed using forward primers: 5' GGCCATCGATGCAGAAGAAGAGGTA GATT3' or 5'GGCCACTAGTGCAGAAGAAGA GGTAGTATT3'. And reverse primers: 5'CCGCTCGAGAAGGAGTGTATTAAGCTTATC3' or 5'GGCCGCGGCCGCAAGGAG TGTATTAAGCTTATC3'. PTB MS2 fusions were cloned using standard techniques into MS2 vector described elsewhere (Wiegand et al., 2002).

UV crosslinking/immunoprecipitation. RNA probes were *in vitro* transcribed (as per manufactures protocol) with T7 RNA polymerase (Ambion) in the presence of [^{32}P]UTP (specific activity of 5000 Ci/mmole) and gel purified. UV crosslinking reaction components were assembled as previously described (Wagner et al., 1999).

PCR amplification and RT-PCR assay of transfected minigenes. PCR from DNA templates, RNA isolation and RT-PCR analysis were performed as previously described (Carstens et al., 2000). Endogenous FGF-R2 was analyzed using PCR primers FGF-FB (Carstens et al., 1998) and IIIcR- 5' AACCTGCAGAGTGAAAGGATATCCCGA3'. Endogenous fibronectin was analyzed using PCR primers FibF- 5'TGGAGTACAATGTCAGTGTTT3' and FibR- 5'CTGGACCAAT GTTGGTGAATCGC3'. Quantifications were performed using ImageQuant as follows: products that contained either the IIIb exon (U-IIIb-D) or the IIIc exon (U-IIIc-D) only were quantified for volume and then corrected for molar equivalents. Percentage inclusion from triplicate experiments was calculated using the following formulas: U-IIIb-D/(U-IIIb-D + U-D) or U-IIIc-D/(U-IIIc-D + U-D)). In fig. 3, the fold change in ratio is calculated as follows: in the case of NS RNAi bar for IIIb inclusion products it is the ratio of inclusion products to skipped products for the NS RNAi treated cells divided by the ratio of the inclusion products to skipped products for the NS RNAi treated cells, hence a value of 1. In the case of the PTB RNAi bar for IIIb inclusion products it is the ratio of the inclusion to skipped products for the PTB RNAi treated cells divided by the ratio of the inclusion products to skipped products for the NS RNAi treated cells.

Cellular Transfections and RNAi. Stable transfections were performed as described previously (Carstens et al., 2000). For RNA interference assays, DT3 and 293T cells were plated to a density of 10^5 cells per well in a 24 well plate (Falcon) on day 1. The following day (day 2), 3 ul

of a 20uM stock of siRNA duplex were mixed into 47ul of Opti-MEM media (Invitrogen). In a separate eppendorf tube, 3 ul of Lipofectamine 2000 (Invitrogen) were resuspended in 12 ul of Opti-Mem and allowed to incubate at RT for 7 minutes. The mixtures were combined and allowed to sit at RT for 25 minutes. After the incubation, 35 ul of Opti-Mem were added and the 100ul mixture was pipetted directly into the well containing 500 ul of DMEM plus 10% Fetal Bovine Serum. On day 3, cells were trypsinized and split into a well of a 6 well plate (Falcon). On day 4, cells were re-transfected using 6 ul of siRNA duplex with 3 ul of Oligofectamine (Invitrogen) as the transfection reagent. On day 5, fresh DMEM plus serum was added to the cells. On day 6, cells were transfected with minigenes using Lipofectamine according to manufactures protocol. The following and final day (day 7), cells were harvested for either Western Blot analysis or RNA isolation.

Western Blot analysis. Cell lysates were made by freeze/thaw in 100 mM Tris-HCl and quantified with Bradford Reagent (Biorad). Western detection was performed using standard techniques described elsewhere (Carstens et al., 2000).

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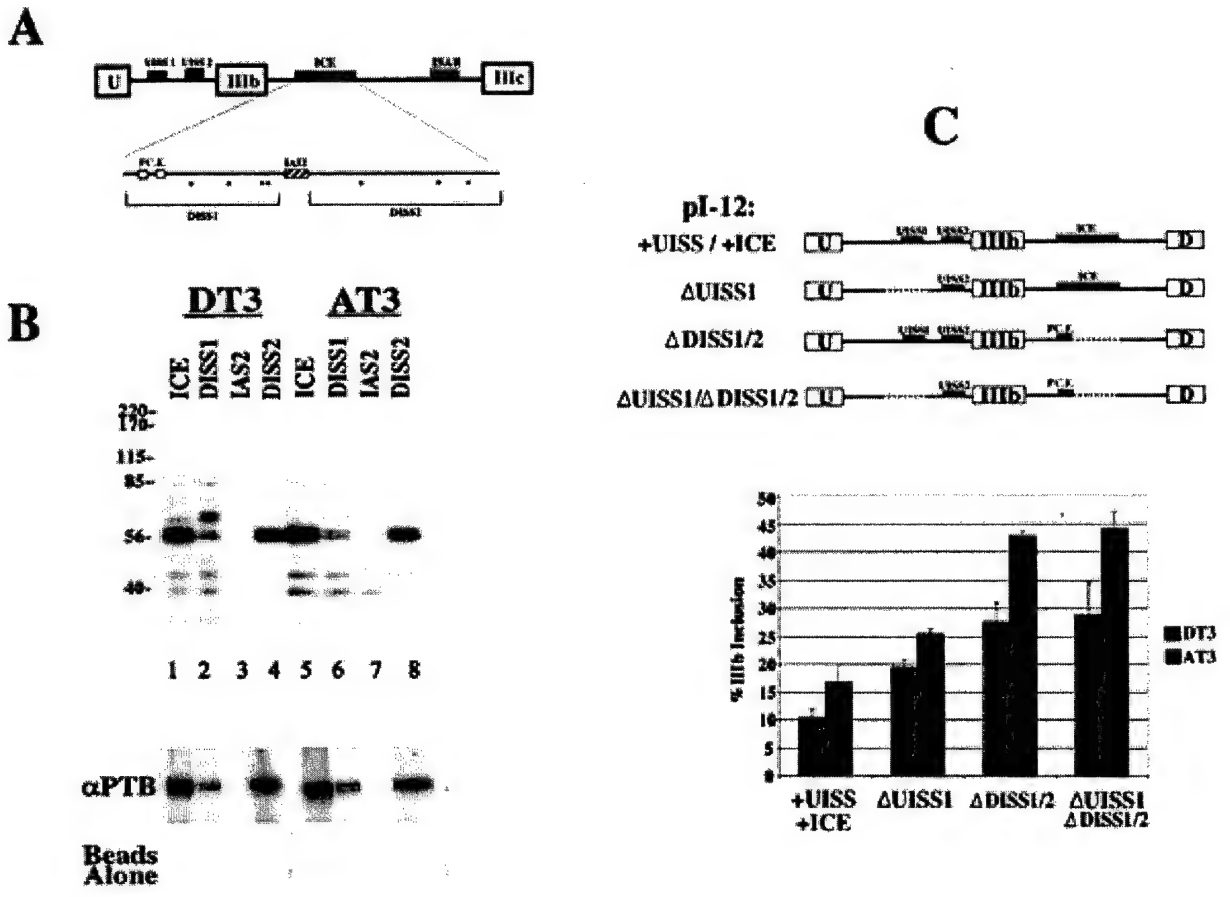

Figure 1. PTB crosslinks to multiple regions of the ICE and the deletion of these binding sites correlates with the loss of exon IIIb repression. **A.** The ICE is broken down into three parts, which were used to *in vitro* transcribe RNA to be used for crosslinking. **B.** Labeled RNA depicted in panel A was incubated in nuclear extract from DT3 and AT3 cells and used for UV crosslinking assays. Aliquots of these crosslinking reactions were then used for immunoprecipitation with anti-PTB antibodies or protein beads alone and are shown in the lower two panels. **C.** Minigenes used to transfect into DT3 and AT3 cells to test the contribution of the PTB binding sites both upstream and downstream of exon IIIb. In the case of DISS1/2 deletions, only the PTB binding sites were deleted leaving the 5' C.E. intact. **D.** Quantification of RT-PCR analysis of stably transfected minigenes in both DT3 cells and AT3 cells.

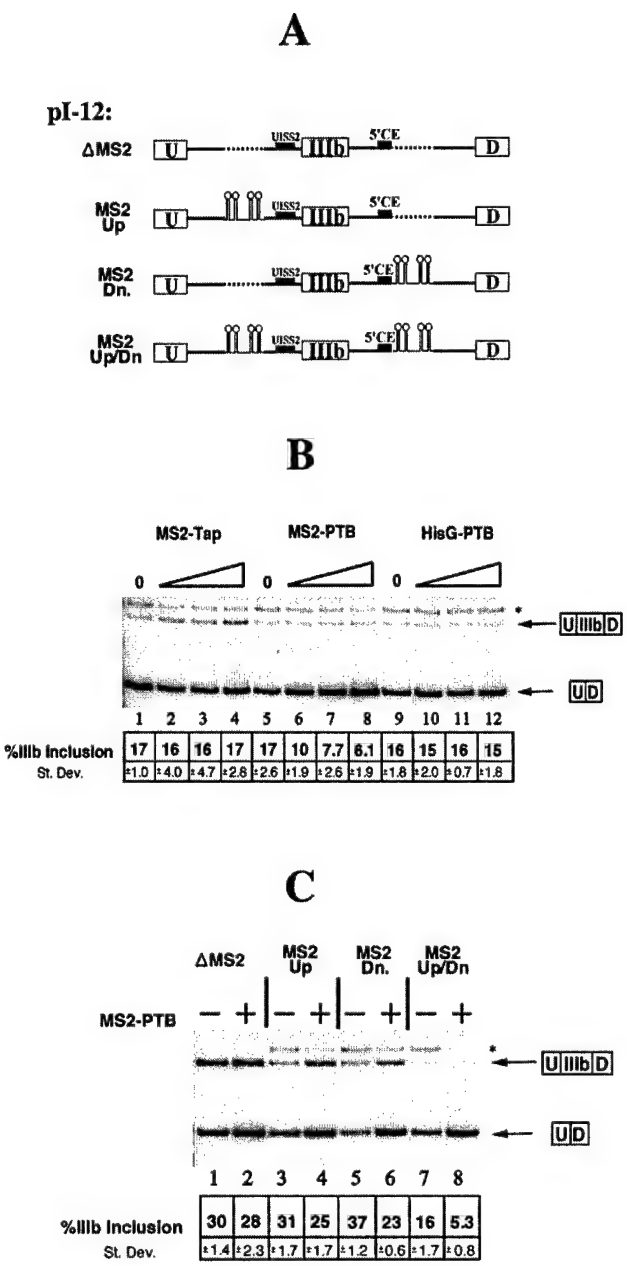

Figure 2. A PTB-MS2 fusion protein silences exon IIIb when recruited upstream or downstream of the exon. **A.** MS2 RNA sequence (4X) was inserted into the deletions of PTB binding sites both upstream and downstream of exon IIIb and used in transient transfections into DT3 cells. **B.** RT-PCR analysis of the co-transfection of MS2 Up/Dn with MS2-Tap, HisG-PTB, or MS2-PTB. A cryptic spliced product, which was also repressed by MS2-PTB, was spliced from a cryptic 3' splice site located at the extreme end of the MS2 stem loop sequence (*). **C.** RT-PCR analysis of co-transfection of denoted minigenes with either MS2 protein alone or 500ng of MS2-PTB.

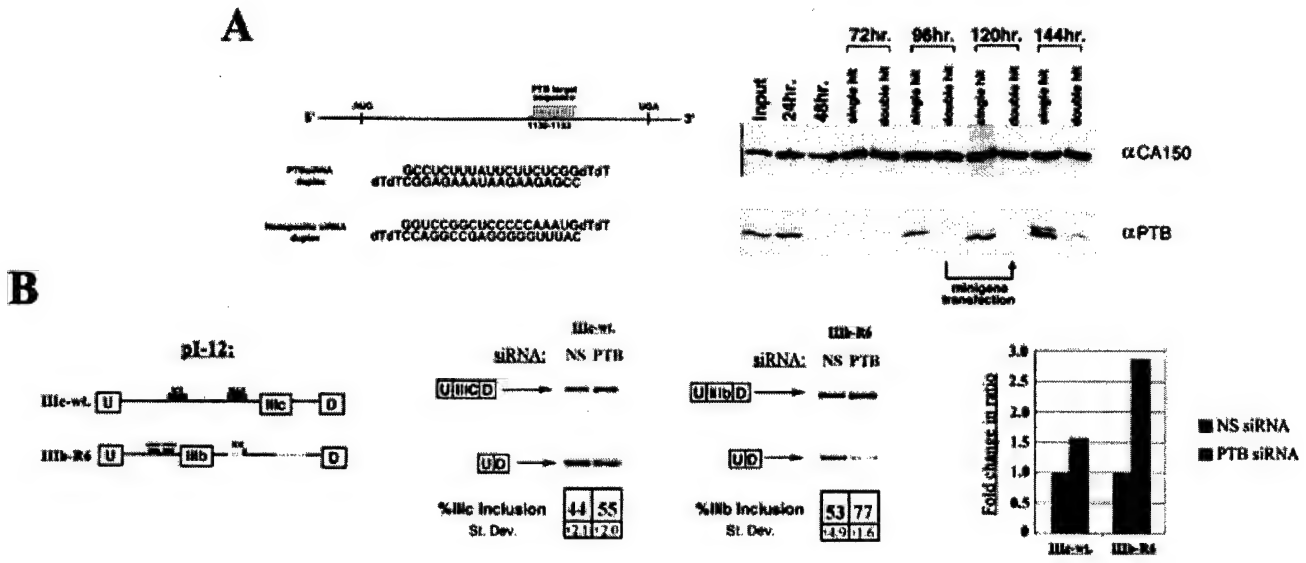

Figure 3. RNAi-mediated *in vivo* depletion of PTB in DT3 cells increases exon IIIb inclusion in FGF-R2 minigenes. **A.** PTB si RNA targets a 19nt. sequence located at nucleotides 1135-1153 of the rat PTB ORF a nonspecific (NS) RNAi duplex targeted the nuclear protein CA150, but did not knock down CA150. In the right panel, DT3 cells were treated with PTB siRNA either once or twice and harvested at 24 hr increments and analyzed for PTB and CA150 expression. **B**. On the left are minigenes used to transfect into DT3 cells treated with either PTB siRNA or NS siRNA. In the middle are the results of RT-PCR analysis of the transient transfection of these constructs into DT3 cells. On the right the RT-PCR analyses are plotted as a ratio of inclusion to skipping relative to the NS siRNA duplex.

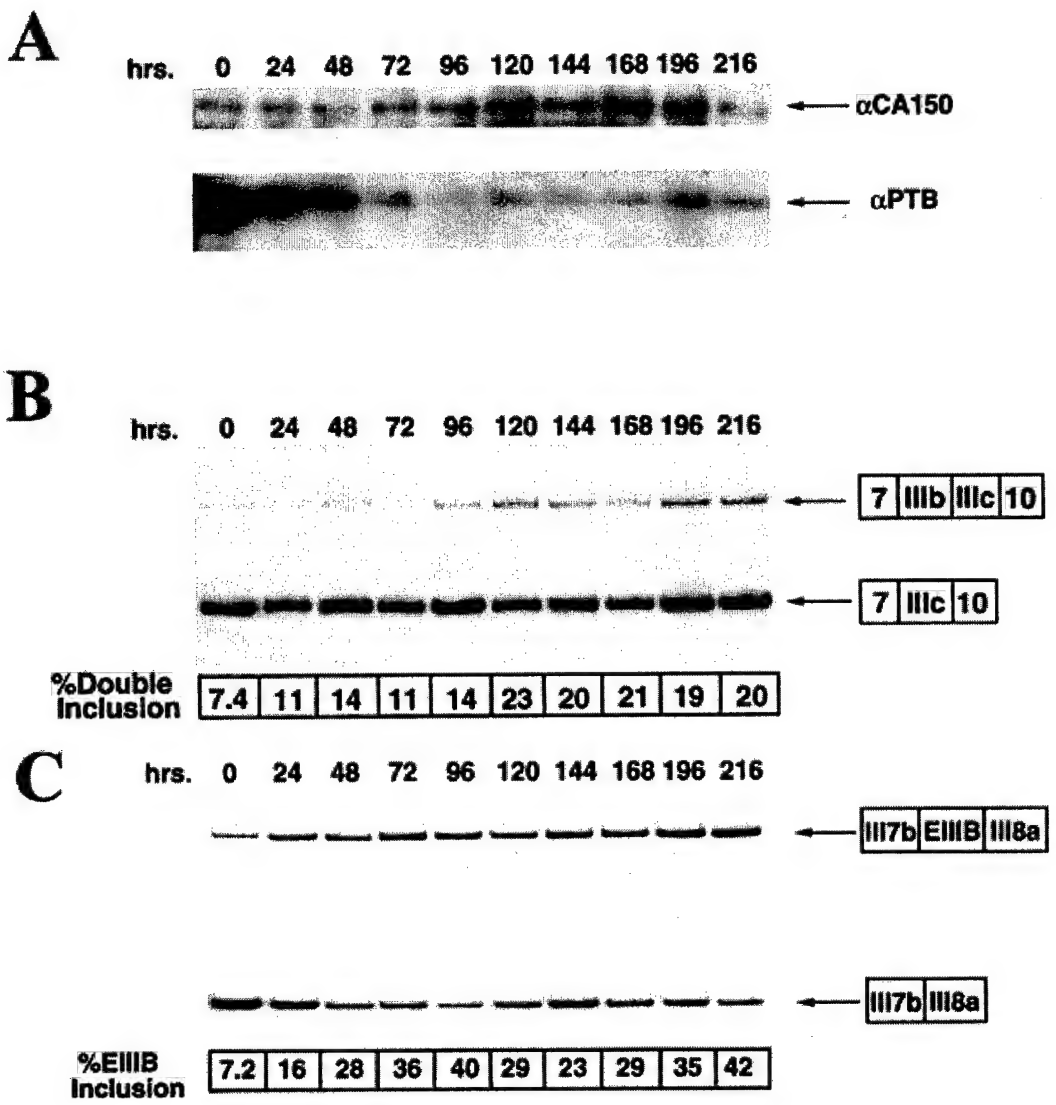

Figure 4. RNAi-mediated *in vivo* depletion of PTB in 293T cells increases the inclusion of FGF-R2 exon IIIb and fibronectin exon EIIIB. **A.** PTB siRNA duplex was transfected into 293T cells at 0 hrs and at 48 hrs. Lysates were collected at the indicated times and analyzed for PTB and CA150 protein expression. **B.** RT-PCR analysis of RNA isolated at each timepoint using primers specific for endogenous FGF-R2. **C.** RT-PCR analysis of RNA isolated at each timepoint using primers specific for endogenous fibronectin.

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Technique

Both Natural and Designed Micro RNAs Can Inhibit the Expression of Cognate mRNAs When Expressed in Human Cells

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Summary

Animal cells have recently been shown to express a range of ~22 nucleotide noncoding RNAs termed micro RNAs (miRNAs). Here, we show that the human mir-30 miRNA can be excised from irrelevant, endogenously transcribed mRNAs encompassing the predicted 71 nucleotide mir-30 precursor. Expression of the mir-30 miRNA specifically blocked the translation in human cells of an mRNA containing artificial mir-30 target sites. Similarly, designed miRNAs were also excised from transcripts encompassing artificial miRNA precursors and could inhibit the expression of mRNAs containing a complementary target site. These data indicate that novel miRNAs can be readily produced in vivo and can be designed to specifically inactivate the expression of selected target genes in human cells.

Introduction

Animal cells have recently been shown to express a novel class of single-stranded, ~22 nucleotide (nt) noncoding RNAs termed micro RNAs (miRNAs) (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). miRNAs appear to be derived from ~70 nt precursors that form a predicted RNA stem-loop structure. It remains unclear whether these miRNA precursors are transcribed from autonomous promoters or are instead contained within longer RNAs (Ambros, 2001; Lau et al., 2001).

While over 100 distinct miRNAs are expressed in organisms as diverse as nematodes, fruit flies, and humans, their function remains uncertain. However, the biological activity of two miRNAs, *C. elegans* let-7 and lin-4, is well established (Lee et al., 1993; Reinhart et al., 2000). Both lin-4 and let-7 are expressed during specific larval stages, and both miRNAs interact with partially complementary RNA targets, located in the 3′ untranslated region (3′UTR) of specific mRNAs, to selectively block their translation. This inhibition is important for appropriate developmental regulation in *C. elegans* (Wightman et al., 1993; Slack et al., 2000).

Several miRNAs, including let-7, are evolutionarily conserved from *C. elegans* to man, as are several let-7 targets (Ambros, 2001). This conservation implies that let-7, as well as other miRNAs, may also repress the expression of specific mRNA species in mammalian cells. This hypothesis is also suggested by the similarity between miRNAs and small interfering RNAs (siRNAs), ~21 nt double-stranded RNAs (dsRNAs) that can induce the degradation of mRNA molecules containing perfectly matched complementary targets, a process termed RNA interference (RNAi) (Sharp, 2001). However, while miRNAs are encoded within the host genome, siRNAs are generally excised from larger dsRNA precursors produced during viral infection or introduced artificially.

Because the introduction of artificial siRNAs into animal cells can induce the degradation of homologous mRNA molecules, RNAi has emerged as a useful experimental tool (Elbashir et al., 2001; Fire et al., 1998; Hammond et al., 2000). However, in mammalian cells, induction of RNAi requires the transfection of RNA oligonucleotides, which can be inefficient and gives rise to only a transient inhibition in target gene expression. It would therefore be invaluable if RNA molecules functionally equivalent to siRNAs could be transcribed from introduced DNA templates.

Here, we first show that an authentic human miRNA, mir-30, can be excised from an irrelevant mRNA transcript encompassing the predicted 71 nt mir-30 precursor (Lagos-Quintana et al., 2001), but not from a transcript containing only the mature mir-30 sequence. Remarkably, these plasmid-encoded mir-30 miRNAs blocked the translation, in human cells, of an indicator mRNA containing predicted mir-30 target sites. Furthermore, we show that novel, designer miRNAs can also be excised from mRNA transcripts containing artificial miRNA precursor sequences and can selectively inhibit the expression of mRNAs containing a complementary target sequence.

Results

Expression of an Introduced mir-30 miRNA Sequence in Human Cells

Mir-30 is one of several novel miRNAs recently isolated from the human cell line HeLa (Lagos-Quintana et al., 2001). We cloned a cDNA sequence encoding the entire predicted 71 nt mir-30 precursor (Figure 1A) into the context of an irrelevant mRNA expressed under the control of the cytomegalovirus immediate early (CMV-IE) promoter, in pCMV-mir-30. We also constructed a similar plasmid, pCMV-mmir-30, containing only the mature mir-30 cDNA sequence. We then transfected human 293T cells with these expression plasmids and analyzed total RNA for the presence of the mir-30 miRNA by Northern blotting (Figure 1B). Mature mir-30 could be readily detected in cells transfected with pCMV-mir-30 (Figure 1B). The miRNA produced from the transfected pCMV-mir-30 plasmid appeared ~22 nt long and had the same 5′ end reported for endogenous mir-30 (Lagos-Quintana et al., 2001), as determined by primer extension analysis (data not shown) (Figure 1A). In contrast, mock-transfected or pCMV-mmir-30-transfected 293T cells expressed no detectable mir-30 miRNA (Figure 1B, lanes 1 and 3). Production of the mir-30 miRNA could also be detected in transfected HeLa or NIH3T3 cells or when the mir-30 precursor DNA was placed within

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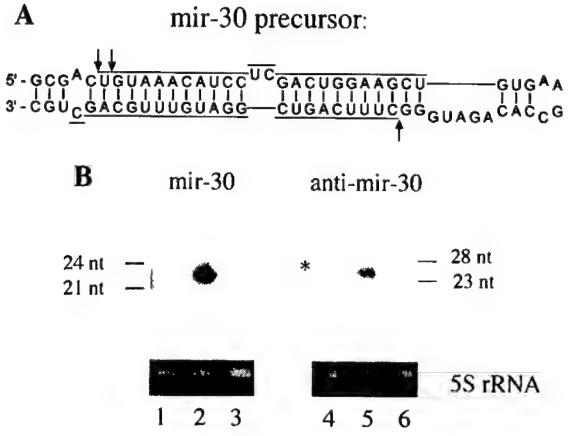

Figure 1. Production of mir-30 miRNA in Transfected Cells

(A) Diagram of the predicted human mir-30 precursor RNA (Lagos-Quintana et al., 2001). Mature mir-30 (3′ arm) and anti-mir-30 (5′ arm) are indicated. Arrows point to the 5′ ends of the mature miRNA as determined by primer extension analysis (data not shown). The position of the 3′ ends may have an error of 1 nucleotide.

(B) Northern blot analysis of mir-30 and anti-mir-30 in transfected 293T cells. Lanes 1 and 4, RNA from mock-transfected cells; lanes 2 and 5, cells transfected with pCMV-mir-30; lanes 3 and 6, cells transfected with pCMV-mmir-30. The relative mobility of synthetic DNA oligos is indicated. "*" indicates the position of a suspected endogenous anti-mir-30 species.

an intron or in the 3′-UTR of another mRNA expressed under the control of the CMV-IE promoter (data not shown). We therefore conclude that the mature mir-30 miRNA can be excised from the mir-30 precursor sequence when the latter is expressed within the context of an irrelevant mRNA.

Mature mir-30 is encoded by the 3′ arm of its precursor (Figure 1A), and one miRNA precursor generally gives rise to only one stable, mature miRNA species, derived from either the 5′ or 3′ arm of the precursor RNA hairpin (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Nevertheless, we were able to also detect a miRNA derived from the 5′ arm (antisense mir-30, or anti-mir-30) in transfected cells (Figure 1B, lane 5). While we did not detect significant levels of endogenous mir-30 miRNA in either 293T or, surprisingly, HeLa cells, there appeared to be a low, constitutive level of endogenous anti-mir-30, or possibly of a similar miRNA, in 293T, HeLa, and NIH3T3 cells (marked by "*" in Figure 1B; data not shown).

Mir-30 Inhibits Expression of an mRNA Containing Complementary Target Sites

The *C. elegans* miRNAs lin-4 and let-7 inhibit the translation of mRNAs containing multiple complementary sequences in their 3′ UTRs without significantly affecting the steady-state level of the mRNA (Lee et al., 1993; Wightman et al., 1993). We therefore asked if human mir-30 could act via a similar mechanism. We designed a mir-30 target sequence and inserted four copies of this sequence into the 3′ UTR of the indicator construct pDM128/RRE to give the pDM128/RRE/4XT plasmid (Figure 2A). Importantly, this target sequence is not a perfect complement to mir-30 and instead, like known lin-4 and let-7 targets (Lee et al., 1993; Slack et al., 2000), contains a central mismatch (Figure 2A).

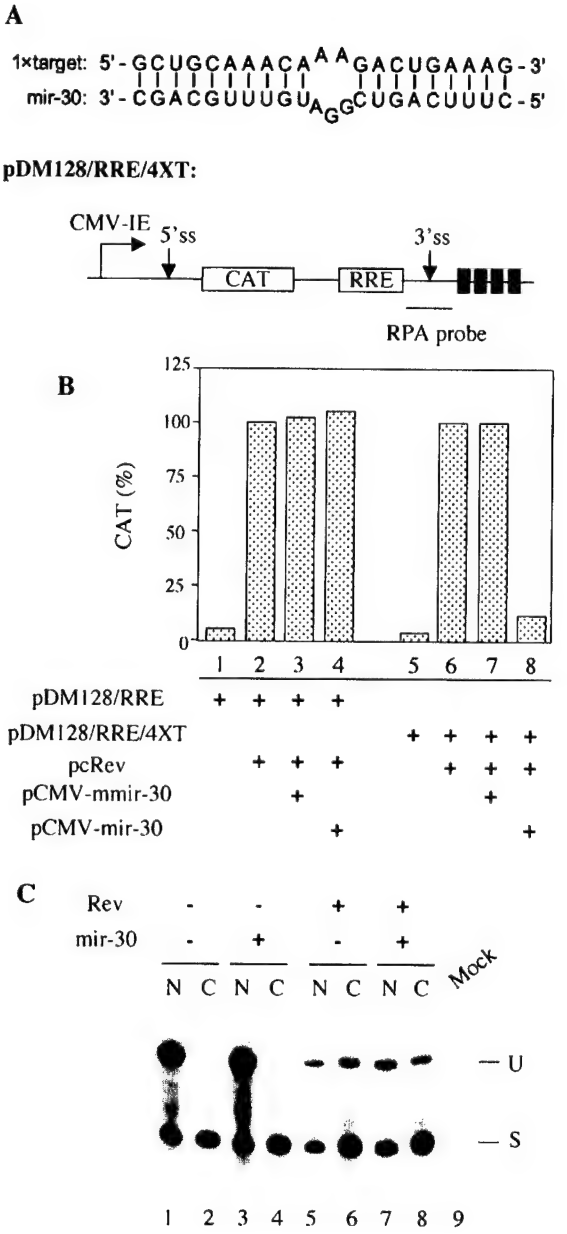

Figure 2. The mir-30 miRNA Selectively Inhibits Expression of an Indicator mRNA Containing mir-30 Target Sites

(A) The sequence of a designed target site partially complementary to mir-30. pDM128/RRE/4XT was derived from pDM128/RRE by insertion of four copies of this target site into the 3′ UTR (black boxes). Splice sites (ss), the RRE, and the relative position of the RPA probe are indicated.

(B) 293T cells were cotransfected with 10 ng of an internal control plasmid (pBC12/CMV/β-gal) expressing β-galactosidase (β-gal) and, as indicated, 10 ng of pDM128/RRE or pDM128/RRE/4XT, 10 ng pcRev, and 400 ng of pCMV-mmir-30 or pCMV-mir-30. The parental pBC12/CMV plasmid served as the negative control. CAT activities were determined at 48 hr posttransfection and were normalized for β-gal activities. Columns 2 and 6 are arbitrarily set at 100%.

(C) 293T cells were transfected with the pDM128/RRE/4XT plasmid, with or without pcRev or pCMV-mir-30, as described in (B). At 48 hr after transfection, cells were divided into nuclear (N) and cytoplasmic (C) fractions, and total RNA was isolated and analyzed by RPA. The probe fragments rescued by the spliced (S) and unspliced (U) mRNAs encoded by pDM128/RRE/4XT are indicated.

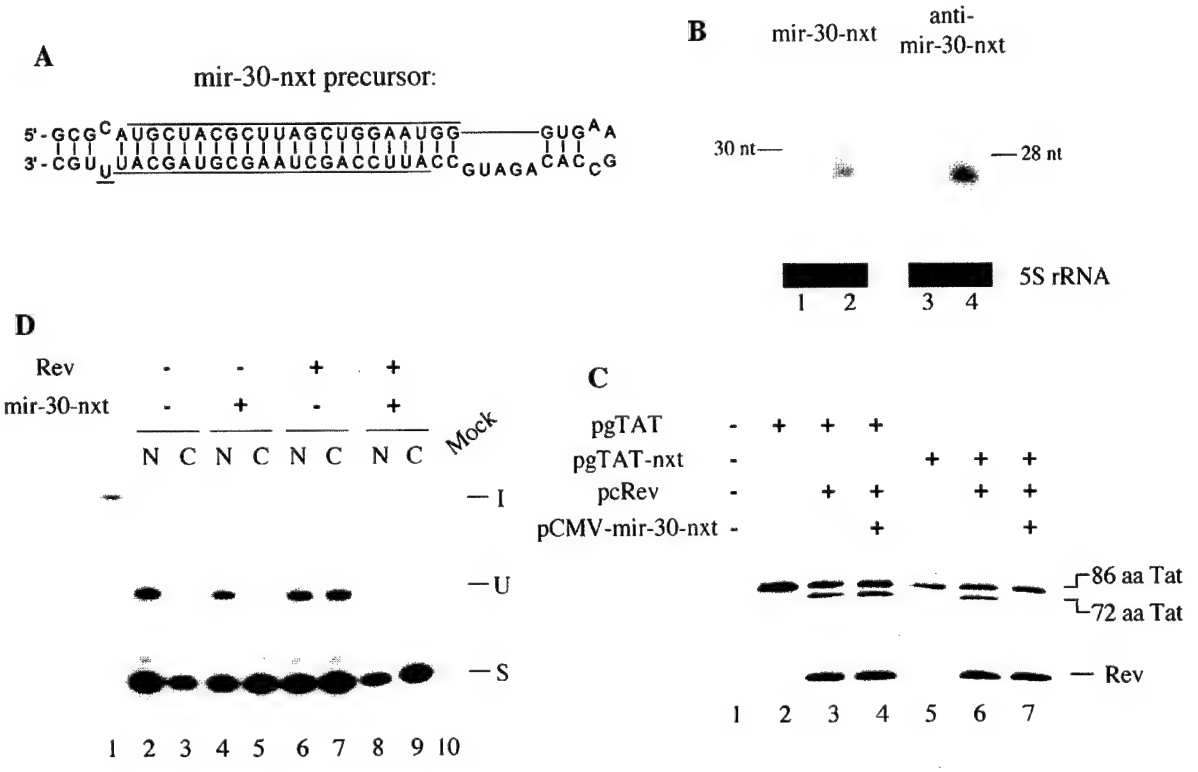

Figure 3. The Novel mir-30-nxt miRNA Specifically Inhibits the Cytoplasmic Expression of Unspliced pgTAT-nxt mRNA

(A) Design of the precursor of the mir-30-nxt miRNA. Inserted sequences derived from the *Drosophila nxt* gene are indicated.

(B) Detection of the novel mir-30-nxt and anti-mir-30-nxt miRNAs in transfected 293T cells by Northern analysis. Lanes 1 and 3, mock-transfected cells; lanes 2 and 4, pCMV-mir-30-nxt-transfected cells. The relative mobility of DNA markers is indicated.

(C) Western blots using rabbit polyclonal antisera directed against HIV-1 Tat or Rev. 293T cells were transfected using 25 ng of pgTAT or pgTAT-nxt, 25 ng of pcRev, and 400 ng of pCMV-mir-30-nxt. The parental pBC12/CMV plasmid served as negative control. This Western analysis was performed ~48 hr after transfection.

(D) The mir-30-nxt miRNA reduces the cytoplasmic level of unspliced pgTAT-nxt mRNA. 293T cells were transfected with pgTAT-nxt, with or without pcRev or pCMV-mir-30-nxt. Two days after transfection, nuclear (N) and cytoplasmic (C) RNAs were prepared and analyzed by RPA. Lane 1 represents approximately 3% of input (I) probe. Probe fragments rescued by spliced (S) and unspliced (U) mRNA are indicated.

The parental pDM128/RRE indicator construct used in these experiments contains 5′ and 3′ splice sites flanking an intron, derived from human immunodeficiency virus type 1 (HIV-1), that contains both the *cat* gene and the Rev Response Element (RRE) (Hope et al., 1990). As previously shown (Hope et al., 1990; Bogerd et al., 1998; Kang and Cullen, 1999), nuclear export of this unspliced *cat* mRNA is dependent on coexpression of the HIV-1 Rev protein, while nuclear export of the spliced mRNA encoded by pDM128/RRE, which does not encode CAT, occurs constitutively (Figure 2). As shown in Figure 2B, cotransfection of pCMV-mir-30, encoding the entire mir-30 RNA precursor, resulted in a marked drop in the level of CAT activity expressed from the pDM128/RRE/4XT plasmid, which contains four copies of the target site, but failed to affect CAT expression from the parental pDM128/RRE indicator plasmid (Figure 2B). In contrast, cotransfection of pCMV-mmir-30, containing only the mature mir-30 sequence, did not reduce CAT expression (Figure 2B).

To determine whether the observed reduction in CAT activity was due to a reduction in *cat* mRNA expression, we performed an RNase protection assay (RPA) using nuclear and cytoplasmic RNA fractions derived from transfected 293T cells. As shown in Figure 2C, mir-30 did not significantly affect the cytoplasmic steady-state level of the unspliced *cat* mRNA encoded by pDM128/RRE/4XT (compare lanes 6 and 8). Thus, the action of the mir-30 miRNA in this reporter system appears to mimic the effect of the lin-4 miRNA in *C. elegans* (Olsen and Ambros, 1999).

Designed miRNAs Can Be Produced In Vivo from Artificial miRNA Precursors

We wondered if we could use the features found in the mir-30 precursor to design and synthesize novel miRNAs in human cells. To this end, we substituted the stem sequence in the mir-30 precursor with a sequence based on the *Drosophila nxt* gene (Gene CG10174, nucleotides 121–143 from the translation initiation codon) (Figure 3A). We have previously shown that analogous synthetic siRNAs can block *nxt* mRNA expression in *Drosophila* S2 cells (Wiegand et al., 2002). Importantly, this sequence is not conserved in human *nxt* homologs.

The new miRNA precursor, termed mir-30-nxt, was again expressed as part of a longer mRNA transcript, as described above for wild-type mir-30. Initially, we transfected the pCMV-mir-30-nxt plasmid into human 293T cells, isolated total RNA, and analyzed the production of both the mature mir-30-nxt miRNA (the 3′ arm,

in accordance with mir-30) and anti-mir-30-nxt (the predicted 5′ arm) by Northern analysis. In Figure 3B (lanes 2 and 4), we show that both mir-30-nxt and anti-mir-30-nxt were indeed expressed. Using primer extension analysis, we determined that the 5′ cleavage sites used in the synthesis of these novel miRNAs were close to those observed in the mir-30 precursor (data not shown). Thus, novel miRNAs can be produced in human cells using the natural mir-30 miRNA precursor as a template.

Inhibition of mRNA Expression by Designed miRNAs

An attractive potential application for designed miRNAs is to use them as siRNAs to initiate RNAi against specific mRNA targets in mammalian cells. As a test of this idea, we constructed an indicator construct, termed pgTat-nxt, containing an inserted 402 nucleotide sequence, derived from the *Drosophila nxt* gene, that should provide a single, fully complementary target site for the novel, mir-30-nxt miRNA. The previously described pgTat indicator construct (Malim et al., 1989) contains the two exons encoding the HIV-1 Tat protein flanking an intron, derived from the HIV-1 *env* gene, that also contains the HIV-1 RRE. In the absence of Rev, pgTat produces exclusively the 86 amino acid (aa), two exon form of Tat encoded by the spliced *tat* mRNA (Figure 3C, lane 2). However, in the presence of the Rev nuclear RNA export factor, the unspliced mRNA encoded by pgTat is also exported from the nucleus, resulting in expression of the short, 72 aa form of the Tat protein (Figure 3C, lane 3) (Malim et al., 1989). Insertion of the *nxt* sequence into the intron of pgTat did not perturb this expression pattern (Figure 3C, lanes 5 and 6). Because the target for pCMV-mir-30-nxt is only present in the intron, expression of mir-30-nxt should only affect the production of 72 aa Tat (in the presence of Rev) but not 86 aa Tat, thus providing an ideal control for specificity. This selective inhibition was indeed observed (Figure 3C, compare lanes 6 and 7). Importantly, mir-30-nxt did not inhibit the synthesis of the Rev protein, of the long form of Tat produced by both pgTAT and pgTAT-nxt, or of the short, 72 aa form of Tat expressed from the pgTAT negative control plasmid (Figure 3C, lanes 4 and 7).

RNAi induces the degradation of target mRNAs (Hammond et al., 2000; Zamore et al., 2000). We therefore performed an RPA to compare the levels of spliced and unspliced Tat mRNAs in the absence or presence of Rev and mir-30-nxt. Mir-30-nxt induced a specific decrease (~7-fold) in the cytoplasmic unspliced *tat* mRNA level seen in the presence of Rev (compare lanes 7 and 9 in Figure 3D), yet had no effect on the spliced *tat* mRNA. Similar results were also obtained using a synthetic siRNA (data not shown), thus strongly suggesting that the mir-30-nxt miRNA is indeed inducing RNAi.

Inhibition of Endogenous Gene Expression Using Artificial miRNAs

To test whether novel miRNAs could inhibit the expression of endogenous genes in human cells, we first chose the polypyrimidine tract binding protein (PTB) (Wagner and Garcia-Blanco, 2001) as a target. The pCMV-mir-30-PTB expression plasmid (containing PTB nucleotides 1179–1201) was constructed as described for pCMV-mir-30-nxt and transfected into 293T cells. Both the mir-30-PTB and the anti-mir-30-PTB miRNA were readily detected by primer extension (data not shown). Importantly, introduction of pCMV-mir-30-PTB resulted in a marked and specific reduction in the level of expression of the endogenous PTB protein and PTB mRNA when compared to control cells (Figure 4A).

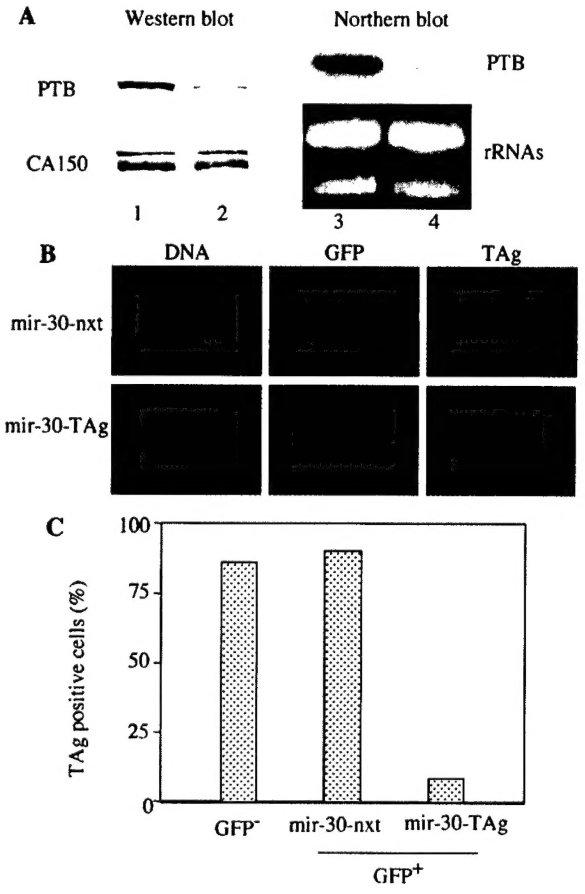

Figure 4. Inhibition of Endogenous Gene Expression by Novel miRNAs in 293T Cells

(A) Reduction of endogenous PTB protein and mRNA expression by pCMV-mir-30-PTB. Cells were transfected with pCMV-mir-30-nxt (lanes 1 and 3) or pCMV-mir-30-PTB (lanes 2 and 4). After 5 days, total cell lysates and RNAs were prepared. Lanes 1 and 2, Western blot using antibodies directed against PTB or CA150, which served as a loading control; lanes 3 and 4, Northern analysis for PTB mRNA.

(B) Loss of SV40 TAg in cells transfected with pCMV-mir-30-TAg. Cells were cotransfected with phrGFP-C and pCMV-mir-30-nxt or pCMV-mir-30-TAg, and 3 days later, analyzed by immunofluorescence.

(C) Quantitation of cells expressing SV40 TAg. Cells with clear nuclear TAg staining were counted as positive (cytoplasmic staining was weak and also present in secondary antibody-only controls). At least 200 cells were counted for each sample.

Although introduction of pCMV-mir-30-PTB resulted in a reproducible 70%–80% drop in the level of PTB protein and mRNA expression in transfected 293T cells (Figure 4A), inhibition was not complete. One possible explanation for the residual level of PTB expression is that transfection of 293T cells is not 100% efficient. To address this question, we constructed a third miRNA expression plasmid, pCMV-mir-30-TAg, designed to ex-

press an artificial miRNA targeted against the SV40 T antigen (TAg) (nt 639–661, Harborth et al., 2001). We then introduced this expression plasmid into 293T cells, which express TAg constitutively, together with a plasmid expressing green fluorescent protein (GFP), and quantitated the number of Tag-expressing cells using immunofluorescence. Cotransfection of the GFP expression plasmid allowed us to readily discriminate transfected from nontransfected cells (Figure 4B). As shown in Figure 4C, ~90% of cells that were not transfected or that were transfected with GFP plus pCMV-mir-30-nxt (as a negative control) expressed readily detectable levels of TAg. In contrast, cotransfection of the pCMV-mir-30-TAg expression plasmid resulted in a dramatic reduction in the number of cells that were both GFP and TAg positive (Figures 4B and 4C).

Discussion

The mir-30 Precursor Is Essential for Production of Mature mir-30 miRNA

Although the production of miRNAs remains poorly understood, one common feature of miRNAs is that they all reside within a putative arm of a predicted ~70 nt precursor RNA stem-loop (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Grishok et al., 2001). Dicer, an RNase III-type enzyme, is believed to be important for the processing of these miRNA precursors into the ~22 nt mature miRNAs (Lee and Ambros, 2001; Hutvágner et al., 2001; Grishok et al., 2001). Importantly, Dicer is also involved in siRNA production from longer dsRNAs (Bernstein et al., 2001; Grishok et al., 2001). At present, it remains unclear why miRNAs are processed only at specific sites and why normally only the sequence from one side of the stem is detected as a mature miRNA, whereas siRNAs are thought to be double-stranded.

We placed the mir-30 precursor sequence under the control of an RNA polymerase II (Pol II)-dependent promoter at various locations in longer mRNAs and observed the production of mature mir-30 in transfected cells (Figure 1 and data not shown). Although this result does not demonstrate that Pol II is responsible for endogenous miRNA transcription, it indicates that miRNAs do not necessarily have to have autonomous promoters and that at least some miRNAs can be processed from longer transcripts. The precursor clearly contains important sequence/structural information for the processing of miRNA, as pCMV-mmir-30, containing only the mature mir-30 sequence, failed to produce any mature mir-30 miRNA (Figure 1B). In fact, these data indicate that at least some miRNA precursors contain all the *cis*-acting sequences required for mature miRNA expression.

The mir-30 produced from our constructs appears to have the same length and 5′ end as reported (Lagos-Quintana et al., 2001) and can inhibit the translation of a reporter mRNA (Figure 2B), thus indicating that it is biologically active. Mir-30 is the third miRNA but the first outside of *C. elegans* to be reported to have the ability to inhibit translation, albeit in an artificial system. While these results do not define the normal function of mir-30 in vivo, they do show that the ability of miRNAs to bind to the 3′UTR of mRNAs and inhibit their translation is evolutionarily conserved.

Given that one miRNA precursor normally gives rise to only one miRNA (Lau et al., 2001; Lagos-Quintana et al., 2001; Lee and Ambros, 2001; Hutvágner et al., 2001), it is surprising that anti-mir-30 was also expressed from pCMV-mir-30 (Figure 1). Judging from the length of the miRNAs and the position of their 5′ ends, 3′ 2 nucleotide overhangs would likely exist if mir-30 and anti-mir-30 were able to anneal, thus potentially implicating dicer in their production. Of note, we also cloned the predicted *C. elegans* lin-4 precursor (Lee et al., 1993), as well as the human mir-27, mir-21, and let-7a3 precursors (Lagos-Quintana et al., 2001), into the same expression vector. Although production of the mir-21 miRNA but not anti-mir-21 was observed, we failed to detect overproduction of the other three miRNAs (data not shown). This suggests that there is a degree of specificity in our system for miRNA production.

Artificial miRNAs Can Function as siRNAs in Human Cells

We have shown, by substituting the stem sequences of the mir-30 precursor with unrelated base-paired sequences, that novel miRNAs can be made in human cells (Figure 3 and data not shown). While this result suggests that the sequence of the precursor stem does not contribute to mature miRNA production, it is plausible that some natural miRNA precursors, such as mir-30, have a relaxed requirement for their stem sequences and thus might be particularly suitable as "vectors" for novel miRNA production.

While exogenously introduced long dsRNAs, or endogenously produced hairpin RNAs, can induce RNAi in various organisms, they have enjoyed only limited success in mammalian systems due to their nonspecific induction of other, less specific inhibitory activities, such as the interferon response (Caplen et al., 2000). An important breakthrough was made when it was shown that synthetic siRNAs can be transfected into mammalian cells and specifically inhibit the expression of target genes, without inducing nonspecific host responses (Elbashir et al., 2001). Here, we demonstrate that designed miRNAs can be produced from transfected DNA in human cells and that these miRNAs can induce the specific degradation of a cognate mRNA target, similar to transfected siRNAs (Figures 3 and 4). Of note, we have recently obtained similar results in HeLa and NIH3T3 cells and have observed that single nucleotide changes in the artificial miRNA, that reduce the complementarity to the mRNA target, attenuate the level of gene silencing observed (data not shown). Therefore, it appears possible to design expression vectors that encode novel miRNAs that can specifically inhibit the expression of mammalian target genes.

This approach offers a number of important potential advantages when contrasted with published protocols that rely on the transfection of synthetic siRNAs (Elbashir et al., 2001). These include: (1) Transfection of miRNA expression plasmids is simple and inexpensive and can result in continuous miRNA production, thus presumably leading to stable inhibition of target mRNA expression. (2) Inhibitory miRNAs could be expressed using viral vectors, thus allowing the production of miRNAs in primary cells or in other cells that are not readily

transfectable with synthetic siRNAs. (3) As the inhibitory miRNA is expressed as part of an mRNA, it should be possible to use regulatable promoters to control miRNA production. (4) It may be possible to generate phenotypic knockouts by constructing transgenic organisms expressing inhibitory miRNAs, possibly in a tissue-specific manner. We propose that the technology described in this manuscript may have the potential to facilitate the biological characterization of many of the >35,000 genes now known to be encoded within the human genome.

Experimental Procedures

Plasmid Construction and Oligonucleotide Description

The expression plasmids pBC12/CMV, pBC12/CMV/β-gal, and pcRev and the indicator constructs pDM128/RRE and pgTat have been previously described (Malim et al., 1989; Bogerd et al., 1998; Hope et al., 1990). A GFP expression plasmid, phrGFP-C, was obtained from Stratagene. To make pCMV-mir-30, the two DNA primers: 5′-TACTCGAGATCTGCGACTGTAAACATCCTCGACTGGAAG CTGTGAAGCCACAGATGG-3′ and 5′-CGCTCGAGGATCCGCAGC TGCAAACATCCGACTGAAAGCCCATCTGTGGCTTCACAG-3′ were annealed, extended using Taq DNA polymerase, cut with XhoI, and cloned into the XhoI site present in pBC12/CMV. To make pCMV-mmir-30, 5′-ATCCCTTTCAGTCGGATGTTTGCAGCT-3′ and 5′-CTA GAGCTGCAAACATCCGACTGAAAGG-3′ were annealed and cloned into pBC12/CMV. To make pDM128/RRE/4XT, four copies of the mir-30 target site (Figure 2A, separated by 2 or 5 nucleotides from each other) were cloned into the XhoI site of pDM128/RRE. To make pgTAT-nxt, *Drosophila nxt* coding sequence (nucleotides 1–420) were amplified from a *Drosophila* embryonic cDNA library and cloned between the two BglII sites present in pgTAT. The pCMV-mir-30-PTB, pCMV-mir-30-nxt, and pCMV-mir-30-TAg expression plasmids were prepared as described for pCMV-mir-30, except that the inserted stem sequences were derived from each target gene.

Cell Culture and Transfection

293T cells were grown as previously described (Bogerd et al., 1998) and were transfected using FuGene 6 Reagent (Roche). CAT assays were performed at 48 hr after transfection, as described (Bogerd et al., 1998). For Western blotting, lysates were fractionated on a 4%–20% SDS-acrylamide gradient gel (Bio-Rad), transferred, and then probed with a rabbit polyclonal antiserum directed against Tat, Rev (Malim et al., 1989), CA150 (Suné et al., 1997), or PTB. Reactive bands were visualized using ECL (Amersham). A polyclonal antiserum specific for human PTB1 was prepared by immunization of rabbits with a purified recombinant fusion protein consisting of glutathione-S-transferase fused to full-length PTB1. Immunofluorescence analyses were performed as described (Wiegand et al., 2002) using a monoclonal antibody against SV40 TAg (Pab 108, Santa Cruz) and rhodamine-conjugated goat anti-mouse antiserum (ICN) as well as the DNA stain DAPI.

RNA Analysis

Total RNA was isolated using Trizol Reagent (Invitrogen). Cell fractionation and RPA were performed as previously described (Kang and Cullen, 1999). For miRNA Northern analysis, approximately 20 μg of total RNA was separated on a denaturing 15% polyacrylamide gel, transferred to a HyBond-N membrane (Amersham), UV cross-linked, and probed with 5′ ^{32}P-phosphorylated oligos in ExpressHyb solution (Clontech). For Northern analysis of mRNA, 20 μg of total RNA was fractionated on a 1% denaturing agarose gel, transferred to membrane, fixed, and probed with a random primed PTB cDNA probe.

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